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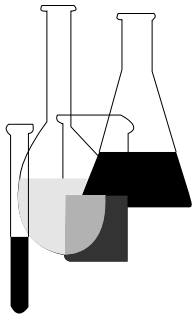
Ecological Effects Test Guidelines

OPPTS 850.4600 *Rhizobium*-Legume Toxicity



"Public Draft"

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202–512–1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202–512–0135 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines.’’

OPPTS 850.4600 Rhizobium-legume toxicity.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is 40 CFR 797.2900 *Rhizobium*-legume Chronic Toxicity Test (proposed in the FEDERAL REGISTER of September 28, 1987 (52 FR 36339)).

(b) **Purpose.** This guideline is intended for use in developing data on the toxicity of chemical substances and mixtures (''test substances''). The guideline prescribes tests using commercially important terrestrial plants and their nitrogen-fixing bacterial symbionts to develop data on the phytotoxicity of test substances. EPA will use data from these tests in assessing the hazard of a test substance to the environment.

(c) **Definitions**. The definitions in section 3 of TSCA and 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply:

EC X means the experimentally-derived test substance concentration that is calculated to affect X percent of the test effect (e.g., EC40).

Germination means the resumption of active growth by a plant embryo.

Legume means a member of the pea family (leguminosae) and includes many species of great economic importance.

Nitrogen-fixation means the conversion of elemental nitrogen to nitrates by *Rhizobium* which colonize legume root nodules.

Rhizobium means a genus of symbiotic bacteria that forms nodules on the roots of certain legumes.

Support media means the quartz sand used to support the plant.

Symbiont means either of two organisms participating in a symbiotic relationship.

Symbiosis means the close union of two dissimilar organisms in a mutually beneficial relationship.

(d) **Test procedures**—(1) **Summary of the test**. Seeds of a legume species are inoculated with their specific *Rhizobium* symbiont and planted in sand irrigated with a nutrient solution. The test substance is applied to the plant-bacteria complex via the nutrient solution or is adsorbed to the support media, resulting in continuous exposure to the test substance from the time the seed (or seedling, if appropriate) is planted to maturity

of the plant. After significant leaf development has occurred (usually after several weeks during which the *Rhizobium*-infected plants are irrigated at regular intervals with the nutrient solution), all plants are harvested for analysis. Effects are evaluated by comparing plant yield, nodule production, and nitrogen-fixation in plants exposed to the test substance to those plants not exposed (negative controls) to the test substance.

(2) **Application of test substance**. (i) Deionized or glass-distilled water should be used in making stock solutions of a water-soluble test substance. Sufficient quantities of each concentration should be made as needed to minimize storage time and disposal volume. A measured portion of the stock solution should be added to the nutrient solution just before beginning the test.

(ii) A test substance that is insoluble in water, but which can be suspended in an aqueous solution by a carrier, should be added with the carrier, to the nutrient solution. The carrier should be soluble in water, nontoxic to plants, and used in the minimum amount required to dissolve or suspend the test substance. There are no preferred carriers—however, acetone, gum arabic, polyethylene glycol, ethanol, and other solvents have been used extensively in testing herbicides, plant growth regulators, fungicides, and other chemical substances that affect plants. Carrier controls should be included in the experimental design and tested simultaneously with the test substance.

(iii) A water-insoluble test substance for which no nontoxic, water-soluble carrier is available should be dissolved in an appropriate volatile solvent. The stock solution of the test substance should be mixed with the support media, placed in a rotary vacuum apparatus and evaporated, leaving a uniform coating of the test substance on the support media. A weighed portion of support media should be weighed, the test substance should be extracted with the same organic solvent, and the concentration of the test substances should be determined before the potting containers are filled. Solvent controls should be included in the experimental design and tested simultaneously with the test substance.

(3) **Selection of initial test substance concentrations**. (i) A preliminary test should be conducted to determine the concentrations of test substance to be used in the definitive test for each *Rhizobium*-legume association. For this purpose, seed germination, the first event in the establishment of a *Rhizobium*-legume symbiotic relationship, may be used.

(ii) If the concentration of test substance to which the *Rhizobium*-legume association is likely to be exposed in nature can be predicted, seeds of the selected legume should be treated with concentrations that are 0.1×, 1×, and 10× the anticipated environmental concentration. After a given exposure period, the effects should be assessed as the sum of the root lengths (in millimeters) of all plants of each test concentration, relative

to that evidenced in the controls. Should reasonable predictions of potential environmental exposure concentrations not be possible, seeds of the same legume should be exposed to a series of widely spaced concentrations (e.g., 0.01, 0.1, 1.0, 10, 100, 1,000 mg/L) of the test substance. After a given period, root lengths should be compared as previously described. The lowest concentration tested in the series, exclusive of controls, should be at the analytical detection limit of the test substance. The upper concentration, for water-soluble test substances, should not exceed 50 percent of the saturation concentration.

(iii) The seed-germination test consists of exposing a minimum of 15 seeds of one legume species (representing the plant host in the selected *Rhizobium*-legume association) to each concentration of test substance and to the control. Seeds, placed between sheets of filter paper moistened with varying concentrations of test substance, should be incubated in darkness at room temperature (approximately 22 °C) in Petri dishes, allowing adequate room for linear root growth. When 65 percent of the control seeds have germinated and developed roots at least 20 mm long, the test may be terminated.

(iv) No replicates are required and nominal concentrations are acceptable.

(4) **Definitive test**. (i) The purpose of the definitive test is to determine whether the test substance is toxic to the selected *Rhizobium*-legume association and, if so, to delineate its concentration response curves and EC50 values for each of three variables of the test system used.

(ii) Since the anticipated fate of the test substance involves soil or soil water, and the mechanism of toxicity depends upon root exposure, the test substance should be applied in nutrient solution to the support media or coated on the support media for water-insoluble test substances for which no nontoxic, water-soluble carrier is available. The test substance should be chemically stable in the nutrient solution.

(iii) Seeds of legume species that are subject to attack by mold (e.g., clovers) may be washed with ethanol before being planted.

(iv) Seeds should be mixed with a small amount of moist commercial peat previously inoculated with the desired *Rhizobium* species. Seeds of some legumes (e.g., *Trifolium repens*, white clover) may be planted immediately in pots (0.3 g dry seed per pot) filled to within 2.5 cm of the top with support media of clean, coarse (0.5 to 1.0 mm diameter) sand, while others (e.g., *Phaseolus vulgaris*, bush bean), once inoculated with *Rhizobium*, should be allowed to germinate for as many as 6 days in darkness between moist paper towels before being planted, one seedling per pot. The support media should be irrigated with the nutrient solution before planting occurs. Unless it is necessary to adsorb the test substance to the

support media, the nutrient solution should contain the desired concentration of test substance.

(v) Six replicate pots of seed for each of at least five concentrations of test substance, exclusive of controls, should be used in the definitive test. For each *Rhizobium*-legume association tested, the concentration range should be selected to define, as closely as possible, the concentration-response curve between the EC10 and EC90.

(vi) Pots should be irrigated regularly (for example, 30 min/h) with nutrient solution, preferably using a system in which water flows from the bottom of the pot upward. Irrigation should be suspended for 6 to 8 h before nitrogen-fixation measurement. Nutrient solution should be replaced with fresh solution at least once every 2 weeks. It may be necessary to replace the nutrient solution more frequently if the test substance is rapidly degraded under test conditions.

(vii) Every test should include controls consisting of the same dilution water, conditions, procedures, bacteria from the same culture, and seed from the same lot used in the exposed groups, except that none of the test substance should be added. If a carrier solvent is needed to dissolve or suspend the test substance, a carrier control should also be included.

(viii) Alternative planting methods may be required when the test substance is highly volatile. An impervious barrier of polyethylene film, a modification of the double pot method, a glass plate, or other appropriate apparatus should be used to prevent volatilization from the root zone. Seeds should be germinated in the dark at approximately 22 °C, and the barrier should be positioned such that the shoots pass through holes in the barrier. A ring of nontoxic, inert, pliable putty should be used to seal the holes around the stems. Control pots should be handled identically to the test pots but with no exposure to the test substance.

(ix) The definitive test consists of exposing the selected *Rhizobium*-legume association to at least five concentrations of the test substance, using a minimum of six replicate pots for each concentration and control, followed by measurements and analyses of nitrogen-fixation, nodulation performance, and plant yield. The duration of the test should be 3 to 7 weeks from the date of planting, depending on the legume used.

(x) Plants should be observed daily. All visible effects of the test substance on plant growth and morphology, such as stunting, discoloration, chlorosis, or necrosis of the leaves should be noted.

(xi) To measure nitrogen-fixation for a small-to-moderate-sized legume species (e.g., *T. repens*, white clover), each pot should be enclosed in an airtight chamber. To enhance the reduction of acetylene (C_2H_2), the chambers may be flushed with an inert gas (evacuating the nitrogen-containing air) before introducing the C_2H_2. Following exposure to C_2H_2 for

a period of time sufficient to yield a linear production of ethylene (C_4H_4), gas samples should be withdrawn and analyzed for C_4H_4 as an index of nitrogen-fixation, using gas chromatography. Should be *Rhizobium*-legume association selected for the test use a larger species of legume (e.g., *P. vulgaris*, bush bean), plant roots may be removed, washed with distilled water, and placed in an airtight plastic jar. Gas samples should be withdrawn and analyzed for C_4H_4 after an appropriate incubation period (as above) in the presence of C_2H_2. Using the test conditions and clover and bean species recommended, incubation periods of 5 h and 1 h, respectively, are suitable for nitrogen-fixation determinations. Optimal incubation times for other species in containers of other sizes may be different.

(xii) Nodulation performance should be assessed by counting the cumulative number of root nodules on the plants from each treatment group. Yield should be recorded as the total dry (70 °C, 48 h) biomass (tops and roots) per pot.

(xiii) The assignment of pots to test substance concentrations should be random. In addition, placement of groups of pots (six per group, all within each group receiving nutrients and test substance from the same source) should be randomized, to the extent possible, in the greenhouse or growth chamber.

(xiv) Irradiation measurements should be taken at the top of the plant canopy and the mean, maximum, and minimum values determined over the plant-growing area. These measurements should be taken daily, but should be taken at least at the start and completion of the test. If the test is conducted in a greenhouse facility, hourly measurements of irradiation should be recorded and presented as daily total irradiance including representative hourly curves for clear-sky conditions and cloudy days.

(xv) Temperature should be monitored continuously at the top of the plant canopy, while humidity should be measured at least once during each light and dark period.

(xvi) For chamber-grown plants, measurements of carbon dioxide concentrations should be made at the top of the plant canopy on a continuous basis.

(5) **Analytical measurements**—(i) **Test substance**. Stock solutions of test substance should be diluted with glass distilled or deionized water to obtain the test solutions. Standard analytical methods, if available, should be used to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured before use.

(ii) **Numerical**. Entire plants (tops and roots) should be dried and weighed, and numbers of root nodules should be counted for the definitive test. Means and standard deviations of ethylene production (from acetylene reduction assay), plant yields, and nodulation should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration response curves.

(e) **Test conditions**—(1) **Test species**—(i) **Selection**. (A) A species of the genus *Trifolium* (e.g., *T. repens*, white clover) is the preferred legume for this test. The specific complementary species of *Rhizobium* should be obtained from a reliable source of bacterial cultures.

(B) As an alternative, other legume species (e.g., *Phaseolus vulgaris*, bush bean) of economic or ecologic importance to the region of impact may be selected for testing. The rationale for selecting alternative species should be provided.

(ii) **Seed selection**. Information on seed lot, the seed year, or growing season collected and germination percentage should be provided by the source of the seed. Only untreated seed (not treated with fungicides, repellents, etc.) taken from the same lot and year or season of collection should be used in a given test. In addition, all seed of a species used in a test should be of the same size class, and that size class which contains the most seed should be selected and used in a given test. Any damaged seed should be discarded.

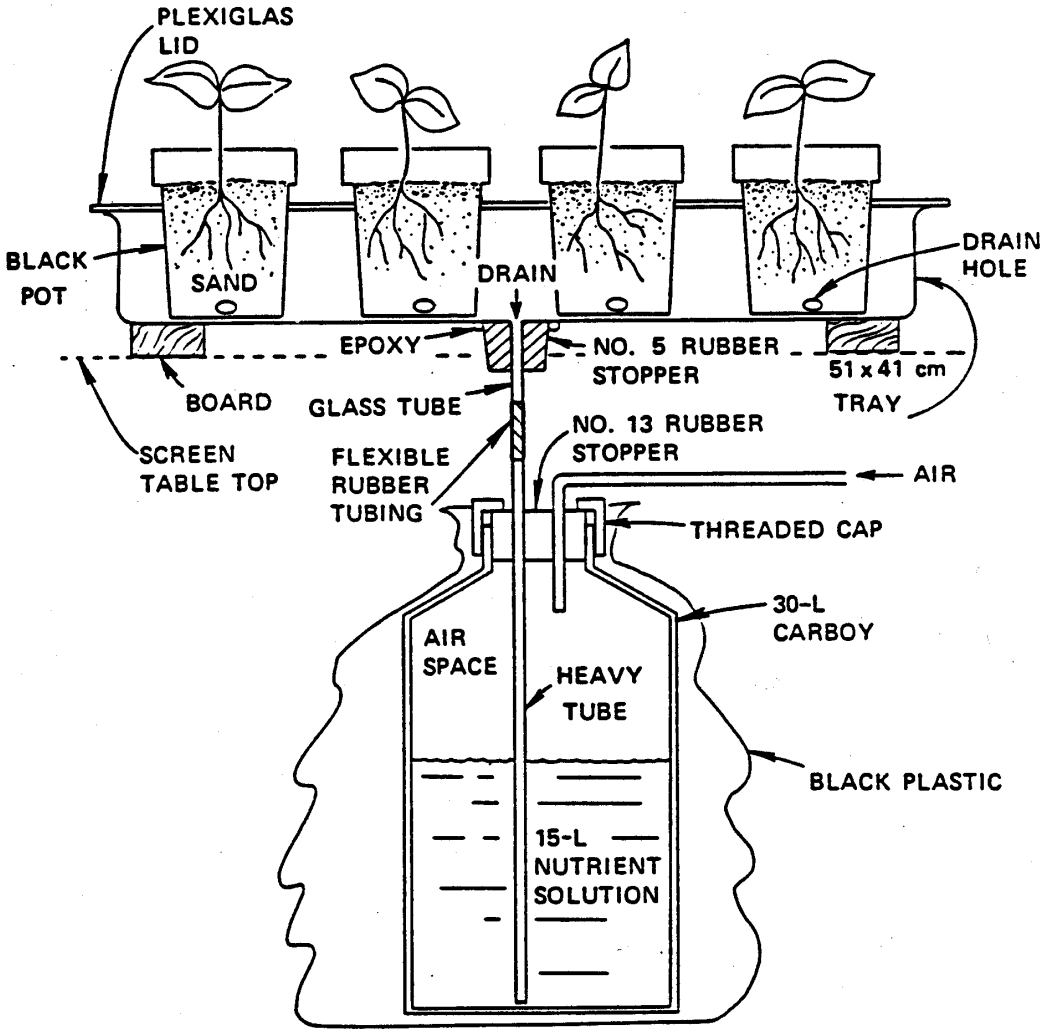
(2) **Facilities**—(i) **Apparatus**. (A) Greenhouse or environmental chambers should provide adequate environmental controls to meet the carbon dioxide, humidity, irradiation, photoperiod, and temperature specifications. Chambers should be designed to prevent escape of internal air into the external environment other than through appropriate filtering material or media to prevent contamination of the external environment with the test substance.

(B) Laboratory facilities for testing should include nonporous floor covering, absorbent bench covering with nonporous backing, and adequate disposal facilities to accommodate plant nutrient, test, and wash solutions containing the test substance at the end of each run, and any bench covering, lab clothing, or other contaminated materials.

(ii) **Containers and support media.** (A) For each run at least 36 to 42 potting containers (6 per concentration of test substance, 6 for the control, and 6 if a carrier control is necessary) will be needed. Containers used in each experiment should be of equal size and volume and possess the same configuration. Potting containers should be filled with support media to within 2.5 cm of their tops. Perlite, vermiculite, native soils, etc. should not be used for root support. A cellulose sponge plug in the pot drain hole will prevent the loss of sand during drainage.

(B) Six or seven 25-L carboys (one per concentration of test substance and one for controls; another if a carrier control is necessary) will serve as reservoirs from which nutrient solution will be delivered, under air pressure, to the appropriate tray of potting containers. An automatic system design is recommended (see the following Figure 1.).

Figure 1.—Cross-Sectional Diagram of a Representative Tray Unit and the Nutrient Solution Reservoir for Irrigating Potted Plants



(C) Each series of six replicate pots (per test concentration, control, and if applicable, carrier control) may be placed in a large tray into which the appropriate nutrient solution will be delivered (see Figure 1. in paragraph (e)(2)(ii)(B) of this guideline). Trays should be constructed of an inert material to which adsorption of the test substance will be minimal, e.g., glass, Teflon, polyethylene, or linear high-density polypropylene. Each tray may be covered with a plexiglas sheet bearing six holes to accommodate the pots, keeping them upright and properly spaced.

(iii) **Cleaning and sterilization.** (A) Potting and receiving containers, nutrient storage containers, and support media should be cleaned before use. All equipment should be washed according to good standard laboratory procedures to remove any residues remaining from manufacturing or

prior use. A dichromate solution should not be used for cleaning pots or other containers.

(B) Support media should be discarded at the end of the test. Disposal should conform to existing regulations.

(iv) **Nutrient media**. (A) The recommended medium for growth and establishment of the *Rhizobium*-legume association consists of the following:

Chemical	Amount (mg/L)
K_2SO_4	901
KH_2PO_4	272
$CaCl_2$	416
$MgSO_4 \cdot 7H_2O$	493
Fe	8.3
H_3BO_3 [1]	2.9
$MnCl_2 \cdot 4H_2O$ [1]	1.8
$ZnSO_4 \cdot 7H_2O$ [1]	0.22
$H2MoO_4 \cdot H_2O$ [1]	0.02
$CuSO_4 \cdot 6H_2O$ [1]	0.03

[1] A single stock solution containing 1,000× concentrations of each of these trace elements should be prepared in advance, so that in use, 1 mL of stock solution per liter of nutrient solution yields the required concentration of each.

For certain legumes (e.g., *Phaseolus vulgaris*, bush bean), growth in this medium will be enhanced by the addition, after 2 weeks, of 50 mL of a nitrate supplement (10.2 g of KNO_3 + 28.3 g of $Ca(NO_3)_2 \cdot 4H_2O$ per liter) to the 15 L of nutrient solution.

(B) The pH of the nutrient medium should be maintained within a range of 4 to 7.

(C) Nutrient solution levels in 25-L carboys should be maintained at 15-L throughout the experiment by replacing transpired water with distilled water, and by complete replacement with fresh solution at least twice a week.

(D) Nutrient solutions should be transported by inert tubing from the carboys to the trays of pots at regular intervals. A timer-activated air pump is recommended for maintaining a controlled irrigation schedule.

(3) **Test parameters**. Environmental conditions should be maintained as follows:

(i) If a growth chamber is used, the carbon dioxide concentration should be 350 ± 50 ppm.

(ii) Irradiation, measured at 1 m from the source, at 350 ± 59 μEinstein/m^2/sec. at 400 to 700 nm.

(iii) Photoperiods of 16 h light and 8 h darkness.

(iv) Optimum temperature for growth and nitrogen-fixation for the species used. For example, the optimum range for clover is 15 to 25 °C.

(v) Relative humidity in growth chambers should approach 70 ± 5 percent during light periods and 90 ± 5 percent during dark periods.

(vi) pH range of 5 to 8.

(f) **Reporting**. (1) The final report should include, but not necessarily be limited to, the following information.

(i) Name and address of the facility performing the study and the dates on which the study was initiated and was completed, terminated, or discontinued.

(ii) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(iii) Statistical methods used for analyzing the data.

(iv) The test substance identified by name, CAS registry number or code number, source, lot or batch number, strength, purity, and composition or other appropriate characteristics.

(v) Stability of the test and, if used, control substances under the conditions of administration.

(vi) A description of the methods used, which should include the following:

(A) Description of greenhouse or environmental chamber conditions, including type, size, and carbon dioxide concentration (applicable to chambers), temperatures, humidity, photoperiod, and lighting intensity.

(B) Description of nutrient solutions including source of any unusual components.

(C) Description of delivery system including a diagram if the design is complex.

(D) Methods used to determine the placement of potting containers in the test trays and the assignment of test concentrations to particular trays of pots to ensure randomization of exposure.

(E) Frequency, duration, and methods of observation.

(vii) A description of the test system used, including the scientific names and sources of the test species (legume and bacterial), and histories of the species (e.g., percentage of plants germinating, seed size class, and culture history of *Rhizobium* strain used).

(viii) The number of total weight (for smaller species) of seeds tested per concentration, number of replicates, description of carriers, any seed sterilization procedures used, and times of exposure.

(ix) Concentration of the test substance in nutrient solution and in the support media when the test substance is soluble in water or solubilized with a carrier; the concentration of carrier solvent in nutrient solution when carrier is used; the quantity of test substance per unit weight of root support media when the substance is coated on the sand.

(x) pH of the nutrient solution when fresh and when replaced. The reported results should include:

(A) The results of the preliminary test and measurements. Species and concentrations of test substance used, and observed effects on seed germination, should be stated.

(B) For the definitive test, the species, concentrations of test substance used, and the following:

(*1*) Mean plant yield (in grams (dry weight) per pot), cumulative nodule count (per pot), and C_2H_4 production (nanomoles per gram (dry weight) per hour and nanomoles per pot per hour) for untreated controls and for each concentration of the test substance used.

(*2*) Visible effects, if any, of the test substance on the intact plants (tops, roots, and nodules).

(xi) A description of all circumstances that may have affected the quality or integrity of the data.

(xii) The name of the sponsor, study director, principal investigator, names of other scientists or professionals, and the name of all supervisory personnel involved in the study.

(xiii) A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis. Results of the analysis of data should include the concentration response curves with 95–percent confidence limits, the results of a goodness-of-fit test (e.g., X^2 test), and EC50's.

(xiv) The signed and dated reports of each of the individual statements or other professionals involved in the study including each person who, at the request or direction of the testing facility or sponsor, conducted

an analysis or evaluation of data or specimens from the study after data generation was completed.

(xv) The locations where all specimens, raw data, and the final report are stored.

(xvi) The statement prepared and signed by the quality assurance unit.